CYTOTOXICITY MEDIATED BY SOLUBLE ANTIGEN AND LYMPHOCYTES IN DELAYED HYPERSENSITIVITY

III. Analysis of Mechanism*

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(Received for publication 31 May 1968)

In previous publications we have described the characteristics (1) and relationship to cutaneous delayed hypersensitivity (2) of an in vitro reaction of sensitized rat lymphocytes. Such lymphocytes kill fibroblasts in the presence of specific antigen. The system has been compared with others considered as in vitro correlates of delayed hypersensitivity (1). In the present paper we report results of experiments designed to elucidate the mechanism by which lymphocytes damage fibroblasts in terms of the changes which are undergone by the cells in the course of the reaction.

Materials and Methods

Methods of sensitization and tissue culture were those previously described (1). For all experiments, lymph node cells harvested from Lewis rats 9 days after sensitization, were used together with 12.5 or 25 μg/ml tuberculoprotein (PPD) (Parke, Davis & Company, Detroit, Michigan). Cytotoxic effects were measured on Lewis and BN fibroblast monolayers 72 hr after addition of lymph node cells or supernatant from lymph node cells incubated with antigen. The surviving fibroblasts were enumerated with an electronic particle counter. For histochemical study 4–6 $\times$ 10^5 fibroblasts were grown on 22 $\times$ 22 mm cover slips in Falcon tissue culture dishes in a moist atmosphere of 95% air, 5% CO_2. 2 days later, the medium was changed and antigen (PPD) and a drop of lymph node cell suspension (0.4 ml) were added. The cultures were incubated a further 24, 48 or 72 hr, and then the cells were fixed for 15 min in three changes of cold neutral buffered formalin. They were then stained for acid phosphatase with the standard azo dye technique (3). They were incubated at 37°C for 15 min in a mixture of sodium alpha-napthyl phosphate, polyvinyl pyrollidone and 0-amino azo toluene in veronal acetate buffer (pH 5.0). The cover slips were washed for 2 min in running water, and mounted in glycerogel.

* This study was supported by United States Public Health Service Grants AI-06112 and AI-06455. The data were presented in part at the meeting of the Reticuloendothelial Society, 6 December, 1967, Winston-Salem, N.C. From a thesis submitted to the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ Supported by United States Public Health Service Training Grant AI-00291.

RESULTS

Enumerative Studies.—The independent action of sensitized lymph node cells. When normal and sensitized lymph node cells were mixed in varying proportions and added to fibroblasts in the presence of specific antigen, fibroblast killing was directly proportional to the number of *sensitized* lymph node cells added (Table I, Fig. 1). It was inferred that sensitized cells do not confer a cytotoxic property on normal lymph node cells in the presence of antigen, but appear to act independently.

TABLE I

The Effect of Mixing Sensitized and Normal Lewis Lymph Node Cells on Lewis Fibroblast Survival

Exp.	Total[a] added lymph node cells	Sensitized lymph node cells	Surviving fibroblasts at 72 hr		Survival at 72 hr (b/a × 100)
			100% Normal lymph node cells	Mixtures contain sensitized lymph node cells	
			a	b	
	× 10^5	%	× 10^5	× 10^5	%
1	50	50	5.8	5.1	90
	100	50	6.9	3.7	54
	150	50	6.6	2.5	38
	200	50	7.2	2.1	29
	25	100	5.8	4.6	78
	50	100	5.8	3.1	53
	100	100	6.9	2.3	33
	150	100	6.6	1.3	20
	200	100	7.2	1.1	15
2	45	67	9.4	7.4	79
	90	67	9.4	7.1	75
	180	67	9.4	7.6	80
	45	100	9.4	6.8	72
	90	100	9.4	7.1	75
	180	100	9.4	5.8	61
3	50	25	6.0	5.0	84
	100	25	5.3	5.0	94
	200	25	6.6	4.6	69
	50	75	6.0	4.1	69
	100	75	5.3	3.5	65
	200	75	6.6	3.4	52
	50	100	6.0	5.0	84
	100	100	5.3	4.1	77
	200	100	6.6	2.6	40

Sensitized lymph node cells from Lewis rats 9 days after injection of 300 μg Tbc.
12.5 μg PPD/ml added to each culture.

Preincubation Experiments.—To determine whether the primary reaction involves an interaction between antigen and sensitized lymphocytes or whether antigen is taken up by fibroblasts, which are then killed by the sensitized cells, sensitized lymph node cells were incubated with PPD in complete medium at 37° C in 95% air, 5% CO_2 in Falcon tissue culture dishes for different periods of time, washed with HBSS three times, and added to fibroblasts which had not been exposed to antigen. Fibroblasts were similarly incubated with PPD and rinsed three times in HBSS; fresh medium and sensitized lymphocytes,

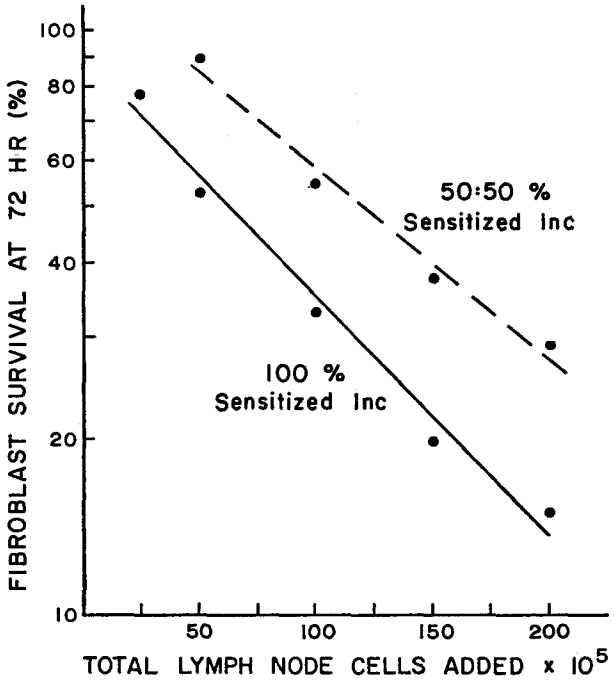

FIG. 1. Cytotoxic effect of mixtures of sensitized and unsensitized lymph node cells (Inc). Graphic representation of data obtained in Experiment 1 (Table I).

which had not been exposed to antigen, were then added. Finally, preincubated, washed lymphocytes were added to fibroblasts which had also been preincubated with antigen, or sensitized lymphocytes and antigen were added to fibroblasts and remained there throughout the experimental period. Control experiments also included normal lymph node cells, or fibroblasts, preincubated with PPD, or sensitized or normal lymph node cells preincubated in the absence of antigen. Cultures were harvested 72 hr after the addition of lymph node cells to fibroblasts. The results (Table II, Figs. 2 and 3) suggest that the mechanism involves an initial reaction of sensitized lymphocytes with antigen, not antigen uptake by fibroblasts. This reaction appears to occur in as short a period as ½ hr. In general, the longer the exposure of lymphocytes to antigen, the lower

TABLE II

Cytotoxicity Following Preincubation of Lymph Node Cells and/or Fibroblasts with PPD

Exp.	PPD concentration	Cells preincubated*		No. of lymph node cells	Length of exposure to PPD	Surviving fibroblasts‡ in presence of		Survival (b/a × 100)
						Normal lymph node cells	Sensitized lymph node cells	
						a	b	
	μg/ml			× 10^5	hr	× 10^5	× 10^5	%
4	0	—	—	150	—	9.3	—	—
	12.5	lnc	—	"	.5	—	6.9	76
	12.5	lnc	—	"	1.0	—	7.3	78
	12.5	lnc	—	"	4.75	—	6.5	70
	12.5	lnc	fbl	"	74	—	5.0	54
5	25	lnc	—	"	0.5	—	4.99	62
	25	lnc	—	"	2	—	5.39	67
	25	lnc	—	"	4	—	4.5	56
	25	lnc	fbl	"	74	8.0	3.8	47
6	25	—	fbl	"	4.5	14.4	12.98	90
	25	lnc	—	"	4.5	14.9	9.8	66
	25	lnc	fbl	"	75	14.0	6.0	43
7	25	—	fbl	100	2.5	5.9	4.4	75
	25	lnc	—	"	2.5	7.4	2.4	32
	25	—	fbl	"	8	9.3	6.7	72
	25	lnc	—	"	8	9.3	1.4	16
	25	lnc	fbl	"	80	9.3	1.3	14
8	0	lnc§	—	—	—	10.5	9.0	85
	25	—	fbl	150	8	10.4	8.7	84
	25	lnc	—	"	8	10.5	4.99	48
	25	lnc	fbl	"	72	8.7	1.96	23
9	0	lnc§	—	110	—	—	5.7	88
	12.5	lnc	—	"	18	—	2.2	34
	12.5	lnc	fbl	"	90	6.5	2.0	31
10	0	lnc§	—	200	—	5.6	2.7	48
	25	—	fbl	"	24	5.6	2.6	46
	25	lnc	—	"	24	4.9	.49	10
	25	lnc	fbl	"	96	5.4	.58	11

* lnc: lymph node cells from sensitized Lewis rats. fbl: Target fibroblasts, BN in Experiments 6 and 7 and Lewis in the remaining experiments.

‡ Cultures harvested within 72 hr after addition of lymph node cells to fibroblasts.

§ Lymph node cells preincubated in medium only.

the fibroblast survival at 72 hr. A maximal effect was observed in some experiments (Experiment 7) with preincubation for 8 hr. The reduced over-all fibroblast survival in Experiment 10 may have been due to the prolonged preincuba-

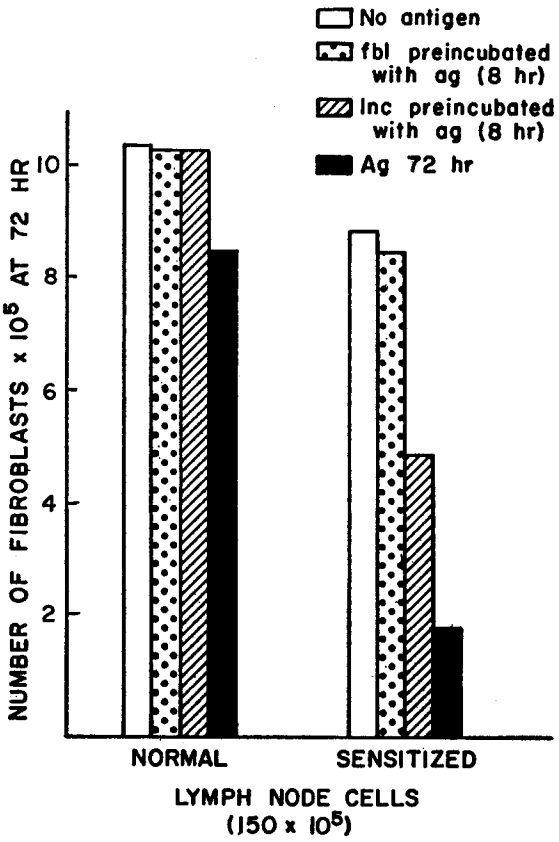

FIG. 2. Cytotoxic effect of sensitized lymph node cells (lnc) after preincubation with antigen. Graphic representation of data obtained in Experiment 8 (Table II). Fibroblasts, fbl; antigen, ag.

tion of an unusually large number of cells, with nonspecific damage to the lymphocytes and release of toxic substances. An apparently specific cytotoxic effect was seen in this experiment nevertheless.

Supernatant Experiments.—Supernatant fluids from lymphocyte suspensions which had been incubated with PPD were spun three times at 900 rpm (250 *g*) to remove cells and, after pH adjustment, were diluted with an equal volume of fresh medium and added to fibroblast monolayers. No attempt was made to

remove antigen from the fluid which was added to the fibroblasts. A cytotoxic effect was obtained with such supernatants after preincubation of cells with antigen for 17 hr or longer (Table III). Supernatants from sensitized lymph node cells preincubated without antigen produced a minimal effect.

Histochemical Observations.—24 hr after the addition of Lewis lymph node cells and PPD, there was no apparent difference in acid phosphatase content between cultures which received normal cells and those to which sensitized lymph node cells had been added. At 48 hr, however, there was a marked in-

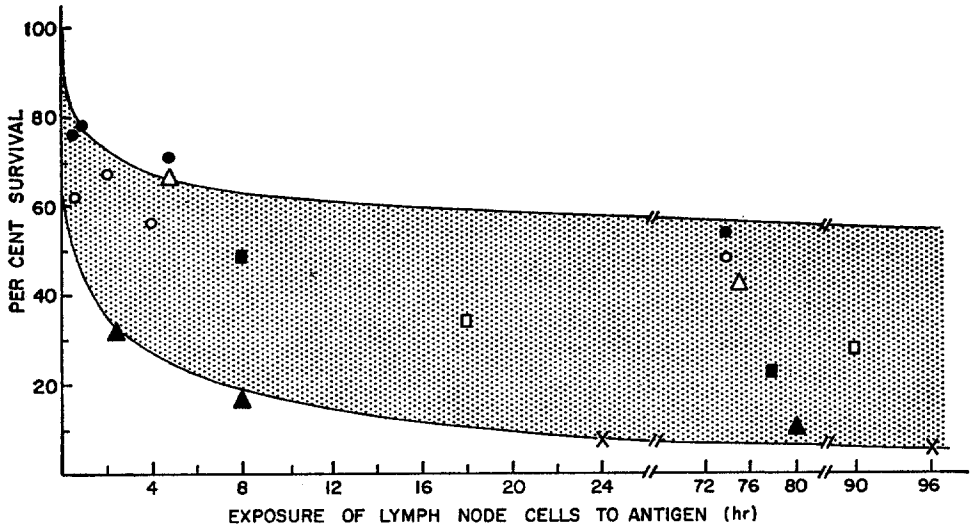

FIG. 3. Fibroblast survival in cultures to which sensitized lymph node cells were added after preincubation with PPD for varying lengths of time. Data from Table II. ● Exp. 4; ○ Exp. 5; △ Exp. 6; ▲ Exp. 7; ■ Exp. 8; □ Exp. 9; × Exp. 10.

crease in the number and size of granules positive for acid phosphatase in fibroblasts of cultures containing sensitized lymphocytes and antigen. Little or no increase in enzyme content was observed in the various control cultures (Fig. 4). At 72 hr, the difference in enzyme content between fibroblasts in experimental and control cultures was even more pronounced. No consistent difference could be seen in the amount of acid phosphatase contained in the lymph node cells. A narrow rim of positive staining material was seen around the nucleus of both "normal" and "sensitized" lymphocytes.

DISCUSSION

Two facets of the phenomenon of in vitro cytotoxicity require consideration here: the role of the lymphocyte in producing the observed cytotoxic effect, and the nature of changes in the fibroblast leading to cell death and the possible

significance of these alterations for other in vivo and in vitro phenomena. Earlier work has established that the fibroblasts are indeed killed (1) and that this phenomenon is related to cellular hypersensitivity (2). The results of preincubation experiments indicate that the cytotoxic effect involves an alteration of sensitized lymphocytes by antigen, *not* a primary uptake of antigen by fibroblasts and a later attack by sensitized cells.

TABLE III

Cytotoxic Effect of Supernatants from Lymph Node Cells Suspensions Incubated with PPD

Exp.	Duration of* preincubation	Surviving fibroblasts in presence of supernatant from			Survival (b/a × 100)
		a	b	c	
		Normal lymph node cells and PPD	Sensitized lymph node cells and PPD	Sensitized lymph node cells alone	
	hr	× 10^5	× 10^5	× 10^5	%
7	2.5	9.09	6.97	—	77
	8	—	3.37	—	37‡
8	8	9.91	9.00	—	91
11	12	11.69	10.59	13.01	91
12	17	12.16	5.72	9.21	47
9	18	5.41	2.8	5.70	52
13	20	3.81	2.06	—	54
	20	3.81	2.15	—	56
10	24	3.88	1.27	—	33

Supernatants were diluted 50% with medium and then added to fibroblasts.

Fibroblasts harvested 72 hr after addition of supernatant.

* 300–450 × 10^5 lymph node cells incubated with 12.5–25 μg PPD/ml for the duration indicated.

‡ Supernatant from normal cells preincubated with antigen for 8 hr was not tested. % survival calculated with regard to survival of fibroblasts in cultures exposed to supernatants from normal lymph node cells incubated with PPD for 2.5 hr.

Berrian and Brent first demonstrated that cells from spleen and lymph nodes of animals with homograft immunity take up transplantation antigen in vitro (4). Turk (5) working with bovine gamma globulin and bovine serum albumin, Kay and Rieke (6) with PPD, and Steffen and Rosak (7) with ovalbumin, showed that specific uptake occurred to a greater extent with sensitized than normal cells and that this uptake was closely correlated with cellular sensitization. Rauch and Raffel (8) found that 5–10% of the small lymphocytes and some medium-sized cells in lymph nodes of guinea pigs with experimental-allergic encephalomyelitis fluoresced after incubation with fluorescein-labeled bovine spinal cord encephalitogen. A number of other authors have published similar findings. However the nature of antigen uptake in these systems, and the fate of the antigen are not clear. Rauch and Raffel (8) state that uptake is not the result

of pinocytosis while Kay and Rieke (6) describe it as an actual incorporation by sensitized lymphocytes, unlike the adsorption of antigen seen with normal nonsensitized cells.

The nature of the alterations which occur in sensitized lymphocytes after interaction with antigen is unknown. Changes in DNA, RNA, and protein (10–16), and the appearance of lysosomal enzymes in lymphocytes (17, 18) during blast transformation following stimulation by phytohemagglutinin have been extensively documented, but it may not be valid to extrapolate these findings to the situation with specifically sensitized cells. Only a small percentage of the lymph node cells from an immunized animal can be specifically sensitized. Alterations in nucleic acids or proteins of the few cells which would exhibit such changes may be undetectable. It is difficult to believe that blast transformation of the lymphocytes as such can be responsible for their cytotoxic action (19), since preincubation experiments show that they release a cytotoxic substance within a few hours while blast transformation takes several days. The sensitized cells apparently do not transfer their specific reactivity to normal lymph node cells (Fig. 1), though substances extracted from such cells are reported to do so (20, 21).

The release of a mediating factor from sensitized lymphocytes, triggered by their reaction with antigen, is strongly suggested by the supernatant experiments. In this, our limited results resemble the findings of David (22) and Bloom and Bennett (23–25) with the capillary tube system for measuring inhibition of macrophage migration. The fact that when sensitized lymphocytes and antigen are placed on a localized portion of a monolayer, fibroblasts in that area are destroyed while those at the periphery of the petri dish appear normal (1) suggests that the substance may not be very diffusible or that fibroblasts may have a strong avidity for it. This finding too is duplicated in the capillary tube system. When two capillary tubes were placed close together in a chamber with antigen, cells failed to migrate from the tube containing sensitized peritoneal exudate, but migrated freely from the adjacent tube filled with unsensitized cells (26). The fact that macrophage-inhibitory factor loses its effect at a dilution of 1:5 or 1:10 could also account for its inability to be effective at a distance (27).

The nature of the cytotoxic substance remains to be defined. Migration-inhibitory factor appears to be protein and its production is inhibited by agents which interfere with protein synthesis (9, 23, 28). In spite of its apparent low molecular weight (25), it may actually be antibody, since it has been reported to act more efficiently in the presence of specific antigen (24). Release of an antibody from the triggered lymphocytes would be comparable to the secondary response of memory cells exposed to antigen. Panayi et al. (29) have described two factors in the supernatant fluid from sensitized lymphocytes incu-

bated with antigen, one behaving chromatographically like antibody and one like albumin or fast alpha-1-protein. The cytotoxic factor may be identical with one of these or may be lysosomal hydrolase(s) released from activated lymphocytes.

The increased acid phosphatase observed in fibroblasts, shortly before their death in cultures exposed to antigen and sensitized lymphocytes, must be a secondary effect of the diffusible substance, discussed above. The cells' increased content of enzymes, which may be described as a form of cell "activation", may nevertheless be responsible for their death under the conditions of the present experiments. Comparable changes have been described in other cell types in experimental situations which involve triggering by specific antigen.

In exudates from sensitized guinea pigs, cultured in the presence of antigen, macrophages increase in size and in their content of phagocytic vacuoles stainable with neutral red (30). These cells usually die over a period of several days but, with increased glucose in the medium, they may remain active (31, 32). Further evidence of their stimulation by specific antigen is their production and release of endogenous pyrogen (33) and interferon (34) and their ability to suppress intracellular replication of bacteria, viruses, fungi, or protozoa to which the host has undergone prior sensitization (35). They are reported to release a substance which in turn stimulates acid production in a variety of normal and neoplastic cells (36). The blast transformation of small lymphocytes, also a form of cell activation, has been shown to be produced by factor(s) released from sensitized cells reacting with antigen (38). Finally, a fibroblastoid transformation is reported to occur in a number of epithelial cell lines when acted upon by the factor released from sensitized human lymphocytes exposed to specific antigen (37).

The concept of "activation", with or without secondary damage or death of the activated cell itself, permits one to account in a simple manner for many of the different target cell effects which have been described (1). Its role in changes occurring in vivo (2) remains to be investigated.

SUMMARY

The cytopathic effect of lymph node cells from tuberculin-sensitized rats on rat embryo fibroblasts in the presence of PPD was not enhanced by admixture of normal (nonsensitized) lymph node cells. Preincubation studies showed that this in vitro response is initiated by the reaction of lymphocytes with specific antigen, beginning within 30 min, rather than uptake of antigen by the fibroblasts. The supernatant fluids from suspensions of sensitized cells incubated with PPD for 17 hr or more possessed cytotoxic activity. The target fibroblasts showed a marked increase in acid phosphatase content within 48 hr after the addition of sensitized lymph node cells and antigen.

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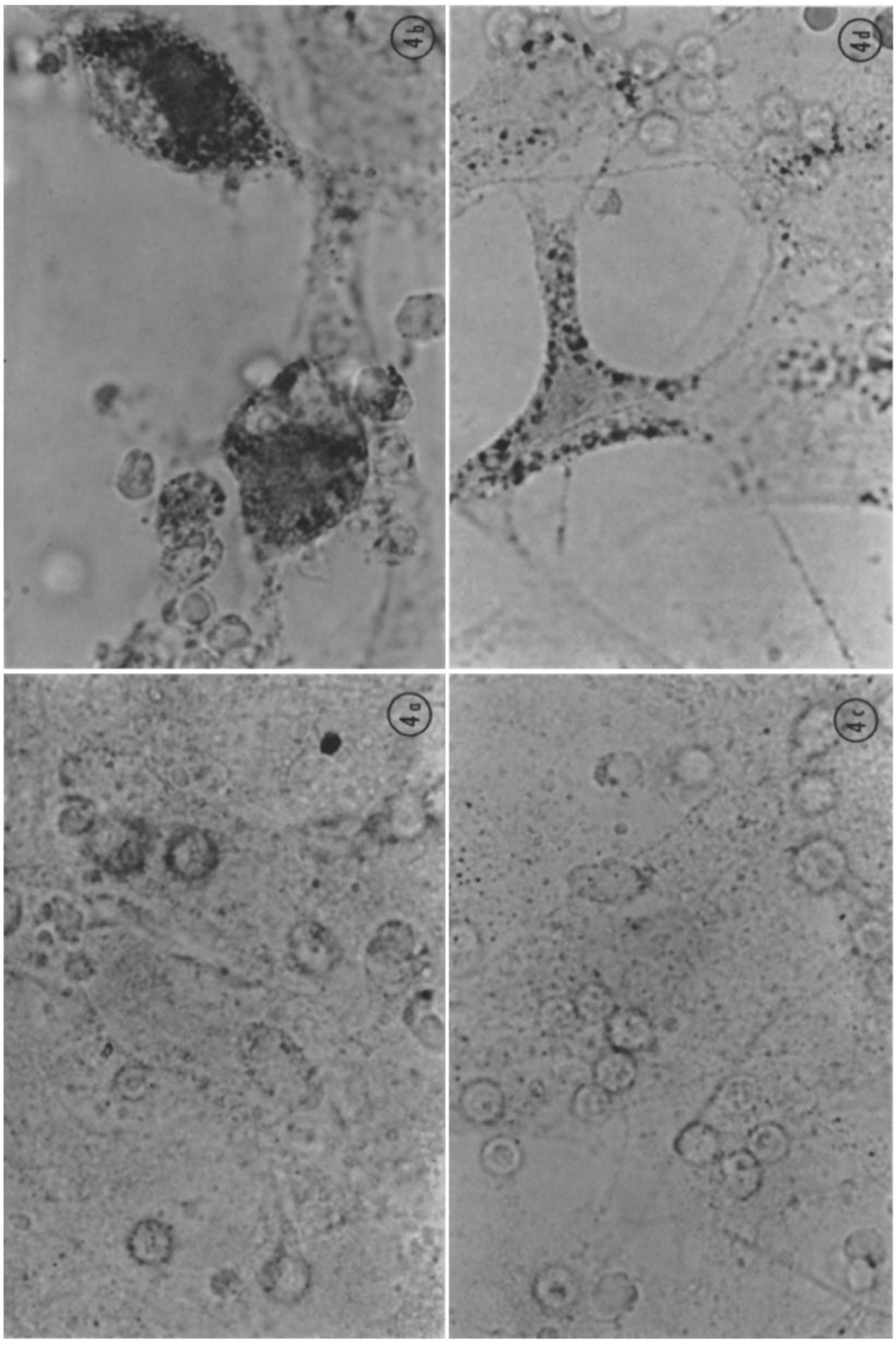
FIG. 4 Histochemical observations. All cells were stained by the azo dye technique for acid phosphatase. Dark granules represent reaction product. ×1250.

FIG. 4 *a*. Lewis fibroblast monolayer 48 hr after the addition of normal Lewis lymph node cells and PPD. No monolayer destruction is evident, and little acid phosphatase.

FIG. 4 *b*. Lewis fibroblast monolayer 48 hr after the addition of tuberculin sensitized Lewis lymph node cells and PPD. The monolayer is damaged and there is much reaction product in the swollen fibroblasts which remain.

FIG. 4 *c*. Lewis fibroblast monolayer 72 hr after the addition of tuberculin sensitized Lewis lymph node cells in the absence of PPD. There is very little acid phosphatase in in the fibroblasts.

FIG. 4 *d*. Lewis fibroblast monolayer 72 hr after the addition of tuberculin sensitized Lewis lymph node cells and PPD. Note the abundant dark granules representing acid phosphatase in the fibroblasts.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